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The goals of this project are to prepare and test novel bifunctional chelates based on DOTA to antibodies directed to CEA (carcinoembryonic antigen) and Her2/neu. The conjugates will be radiolabeled with  $^{111}\text{In}$  for tumor imaging and  $^{90}\text{Y}$  for tumor therapy. In the first year of the project we have shown that the CEA positive MCF7 cell line transfected with Her2/neu can be grown in nude mice injected with estrogen pellets and used as a tumor model. Since we have previously conjugated anti-CEA antibody T84.66 with DOTA and shown it to target CEA positive tumors, we began our work with the anti-Her2/neu antibody 4D5. First, 4D5 was shown to stain the MCF7/Her2/neu cells (>75%). Second, 4D5 was conjugated to DOTA and shown to retain full immunoreactivity (>95%) when radiolabeled with  $^{111}\text{In}$ . Third,  $^{111}\text{In}$  labeled DOTA-4D5 targeted MCF7/Her2/neu tumors in nude mice with excellent kinetics and tumor to blood ratios. Fourth,  $^{90}\text{Y}$  labeled DOTA-4D5 was shown to cause tumor growth inhibition vs untreated, cold antibody or  $^{90}\text{Y}$  labeled irrelevant antibody controls in the same animal model. Studies are in progress to test the efficacy of cold 4D5 plus  $^{90}\text{Y}$  labeled DOTA-4D5. Future studies will test the efficacy of new chelates and the combination of radiolabeled anti-CEA antibody T84.66 plus 4D5.

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## U.S. Army Medical Research and Materiel Command Animal Use Report

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## Definitions of Column Headings on Back of Form

A. Animal	B. Number of animals purchased, bred, or housed but not yet used	C. Number of animals used involving no pain or distress	D. Number of animals used in which appropriate anesthetic, analgesic, or tranquilizing drugs were used to alleviate pain	E. Number of animals used in which pain or distress was not alleviated	F. Total Number of Animals (Columns C+D+E)
Dogs					
Cats					
Guinea Pigs					
Hamsters					
Rabbits					
Non-human Primates					
Sheep					
Pigs					
Goats					
Horses					
Mice	0	0	180	0	180
Rats					
Fish					
List Others:					

\*AAALAC - Association for the Assessment and Accreditation of Laboratory Animal Care

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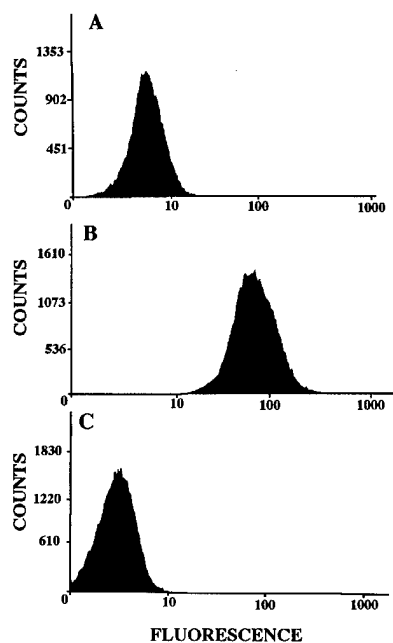
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## Introduction

Breast cancer can be targeted with radiolabeled anti-tumor antibodies. In this project we chose anti-CEA antibody T84.66 for targeting CEA positive breast cancers (about 50% are CEA positive) and anti-Her2/neu antibody 4D5 for Her2/neu positive breast cancer (about 30% are Her2/neu positive). Both antibodies are well characterized and have been used clinically, chimeric T84.66 and humanized 4D5 (Herceptin). The novel aspects of this project are the use of novel chelates to improve the biodistributions and tumor to blood ratios of the radiolabeled antibodies. The radioisotopes are  $^{111}\text{In}$  (2.8 day half life, pure gamma emitter) for imaging and  $^{90}\text{Y}$  (64h half life, pure beta emitter) for therapy. This is a preclinical study to determine the optimum chelate for each antibody. The animal model is a CEA positive/Her2/neu positive cell line grown as a xenograft in nude mice. We propose to test both antibodies separately and in combination.

## Body

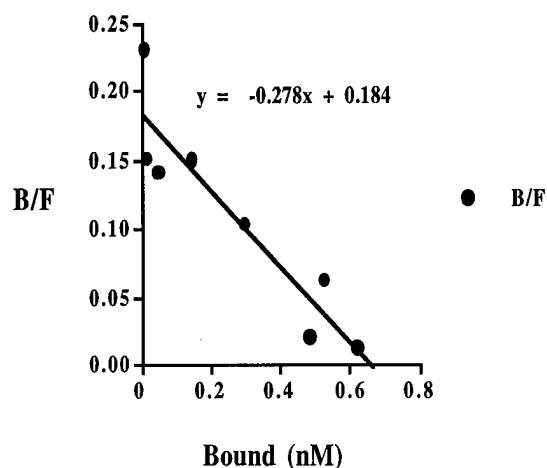
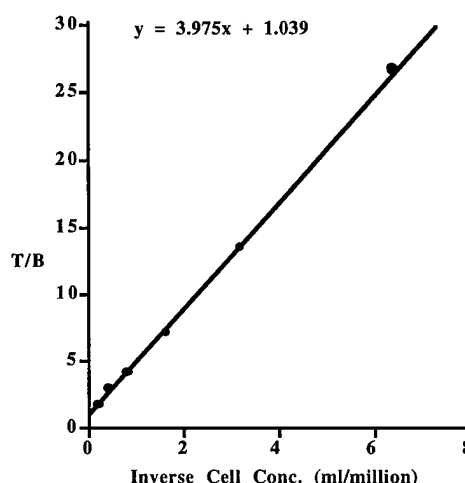
**Cell line.** The MCF7/Her2/neu cell line was obtained from Dr. Dennis Slamon at UCLA. The cell line is positive for both CEA and Her2/neu which was transfected into the parent line MCF7 (1). These cells were analyzed for Her2/neu by FACS using the 4D5 antibody. The results (Figure 1) show intense staining for Her2/neu compared to the parent cell line. The number of Her2/neu sites and binding constant for the antibody was calculated by Scatchard analysis using  $^{125}\text{I}$  labeled 4D5 (Figure 2). These results demonstrate that the cell line is positive for Her2/neu and reacts adequately with the 4D5 antibody.



**Figure 1. FACS analysis of MCF7/Her2/neu cells with anti-Her2/neu antibody 4D5.** A. Parental line MCF7 stained with 4D5 (approximately 6% positive) compared to no primary antibody control (not shown). B. MCF7/Her2/neu cells stained with 4D5 (75% positive compared to no primary antibody control). C. MCF7/Her2/neu cells stained with control antibody Leu16 (93% positive compared to no primary antibody control).

**Antibody conjugate.** 4D5 was conjugated to DOTA (1,4,7,10-tetraazacyclododecane- $\text{N,N',N'',N'''}\text{tetraacetic acid}$ ) using our previously published active ester method (2). Briefly, the antibody (2 mg in 1 mL of PBS) was mixed with EDC (1-ethyl-3-[3-dimethylamino)propyl] carbodiimide) and sulfo-N-hydroxysuccinimide at a ratio of 1:100 for 1 h at room temperature and then dialyzed into 0.2 M ammonium acetate pH 5 buffer. The DOTA conjugated antibody was radiolabeled with either  $^{111}\text{In}$  or  $^{90}\text{Y}$  in the ammonium acetate buffer for 1 h at  $43^\circ\text{C}$ . The radiolabeled antibody was separated from free isotope after the addition of 10 mM DTPA (diethyltriaminopentaacetic acid) by gel filtration chromatography (TosoHaas TSK G2000, 10  $\mu\text{m}$ , 7.5 x 300 mm) in normal saline at a flow rate of 0.5 mL/min and monitored by A280 nm and radioactivity. Based on this analysis, incorporation of radioisotope was 80%. The number of chelates per antibody (5.0) was determined by using radiotracer tagged  $^{111}\text{InCl}_3$ . The immunoreactivity of the radiolabeled antibody was shown to be 95% based on a cell binding assay (Figure 3). Based on these analyses, we conclude that 4D5 can be conjugated to DOTA without loss of immunoreactivity and can be efficiently radiolabeled with either  $^{111}\text{In}$  or  $^{90}\text{Y}$ .

Scatchard plot of 4D5 binding to MCF7-Her2

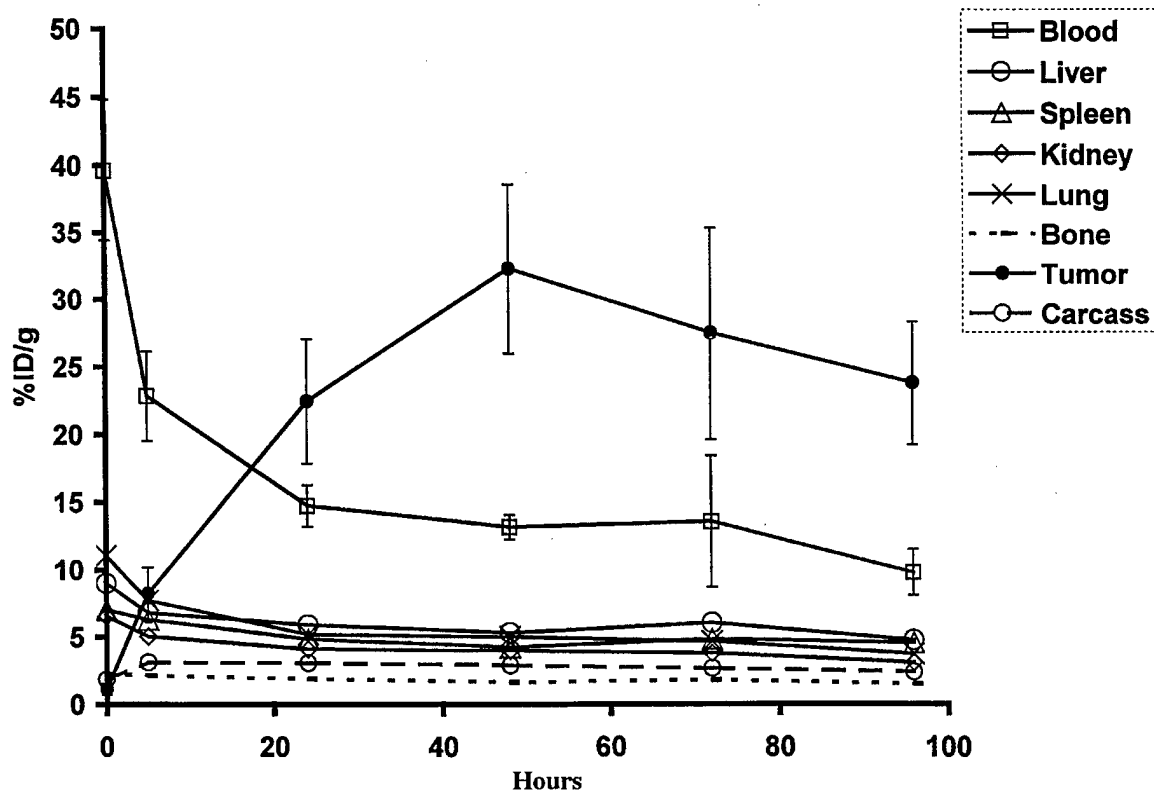
Immunoreactivity plot for  $^{111}\text{In}$ -DOTA-4D5

**Figure 2. Scatchard plot analysis for 4D5 binding to MCF7/Her2/neu cells.** 4D5 was labeled with  $^{125}\text{I}$ , incubated with MCF7/Her2/neu cells at increasing concentration of antibody and the fraction bound over free plotted against total bound.

**Figure 3. Immunoreactive fraction of  $^{111}\text{In}$  labeled DOTA-4D5.** Labeled 4D5 was bound to MCF7/Her2/neu cells, and the fraction of total counts over bound was plotted against the reciprocal of the number of cells. The y-intercept corresponds to % immunoreactivity (95%).

**Animal biodistributions.** Groups of five nude mice received 1.7 mg, 60d release  $^{17}\beta$ -estradiol pellets (sc in the shoulder pads) 3 d prior to inoculation with tumor ( $10^6$  cells, sc in the flank). Once tumors were visible (12d), they were injected with 2.8  $\mu\text{Ci}$  of  $^{111}\text{In}$  labeled DOTA-4D5. Animals were sacrificed at 0, 5, 24, 48, 72, and 96h post injection, tissues removed, weighed, and counted. The results shown in **Figure 4**, demonstrate a maximum tumor uptake at 96h (24 %ID/g) with a tumor to blood ratio of 2.4. Accumulation in the liver which reached 11%ID/g at 48h is in line with our previous studies using chT84.66 (3). There was negligible uptake in other organs such as lung, spleen, and kidney. These results demonstrated that the radiolabeled antibody could target to tumor and result in clinically useful tumor to blood and tumor to liver ratios.

**Radioimmunotherapy in the animal model.** Using the same model, 50 or 100  $\mu\text{Ci}$  of  $^{90}\text{Y}$  labeled DOTA-4D5 was administered to groups of 9 animals. Control groups were injected with 100  $\mu\text{Ci}$  of  $^{90}\text{Y}$  labeled DOTA-Leu16 (irrelevant antibody), unlabeled DOTA-4D5 (3  $\mu\text{g}$ , the same amount of antibody as in the radiolabeled group) or saline. Tumor volumes ( $L \times W^2/2$ ) were measured twice weekly and relative tumor volumes calculated (compared to RIT at day 1). The results (**Figure 5**) show that mice injected with 50  $\mu\text{Ci}$  of  $^{90}\text{Y}$ -labeled DOTA-4D5 had a two-fold

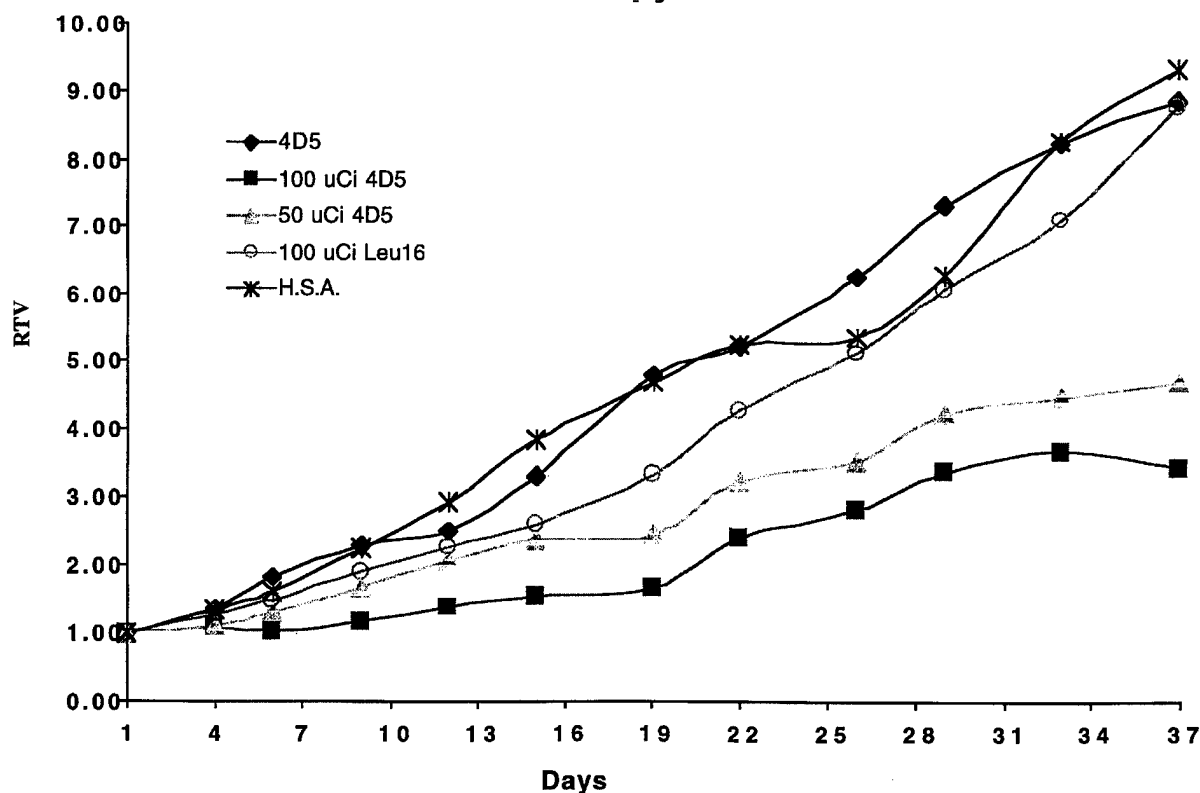


**Figure 4. Biodistributions of <sup>111</sup>In labeled DOTA-4D5 in MCF7/Her2/neu tumor bearing nude mice.** The mice have 60d estrogen implants and were injected with 2.8 uCi of <sup>111</sup>In labeled DOTA-4D5. Animals were sacrificed at the indicated time points, tissues removed and counted. Error bars represent mean of five animals at each time point.

reduction in tumor growth compared to control groups at the end of 37d. For those treated with 100 uCi of radiolabeled antibody, tumor growth was reduced 2.7 fold. Since this was only a single injection, tumor regrowth started to occur at 19d. There were no tumor cures with the single injection of <sup>90</sup>Y labeled DOTA-4D5.

**Conclusions.** The results confirm that the animal model is suitable for evaluating radioimmunotherapy of <sup>90</sup>Y labeled DOTA-4D5. We are now in a position to compare these results to other chelates conjugated to 4D5 and to <sup>90</sup>Y labeled DOTA-T84.66 (anti-CEA antibody). Before beginning these studies we have decided to test the efficacy of cold 4D5 plus radiolabeled 4D5. The reason for performing these studies is their potential clinical significance; i.e., Herceptin plus chemotherapy is currently being used to treat Her2/neu positive breast cancer patients in the clinic. We reason that Herceptin (cold) plus <sup>90</sup>Y radiolabeled DOTA-Herceptin would have certain advantages over the current regimen. If we confirm that cold 4D5 plus hot 4D5 is effective, we will perform biodistribution studies with radiolabeled Herceptin and file an IND to start human studies. Thus, these preclinical studies will have a direct impact on our ongoing human studies.





**Figure 5. Radioimmunotherapy of MCF7/Her2/neu tumors with  $^{90}\text{Y}$  labeled DOTA-4D5.** Five groups of nine animals each were treated as indicated in the legend: Group 1: 2 ug of unlabeled antibody 4D5. Group 2: 100 uCi of  $^{90}\text{Y}$  labeled DOTA-4D5 (2 ug). Group 3: 50 uCi of  $^{90}\text{Y}$  labeled DOTA-4D5 (2 ug). Group 4: 100 uCi of  $^{90}\text{Y}$  labeled DOTA-Leu16 control antibody (2 ug). Group 5: 2 ug of human serum albumin in normal saline. Relative tumor volumes were calculated at day 1 of therapy and average among the nine animals.

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